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On the Toxic Value of Certain Salts of Mercury and Copper

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WITHDRAWN

ON THE TOXIC VALUE OF MERCURIC CHLORIDE AND ITS DOUBLE SALTS

BY JUDSON F. CLARK

The toxic properties of mercuric chloride have been known since time immemorial. Its special value as a germicide has been recognized by the biological and medical professions ever since the publication of Koch's[1] classic work in 1881. Quite a large amount of work has been done on it from time to time since that date, the general results of which have confirmed the conclusion arrived at by Koch that mercuric chloride is one of the most powerful poisons known for all forms of animal and vegetable life. It has also been shown that while the lower forms of life, such as putrefying and pathogenic bacteria, are particularly susceptible to it, its value as a disinfectant is in many cases of practical sanitation greatly impaired, and may even be entirely destroyed by the presence of an excess of albuminous matters in the material to be disinfected, and its affinity for such substances, whether dead or living. In the chemical reaction which results the mercuric chloride as well as the proteid is precipitated in the form of an extremely insoluble and consequently harmless mercury albuminate.

In an endeavor to overcome this difficulty in practical use, investigators have studied the effect of adding various substances to the pure $HgCl_2$ solution. The substances most frequently added have been the chlorides of the alkalies and other metals, iodides, bromides, and other salts, such as potassium cyanide, and sulphocyanide, sodium nitrate, etc.; also various acids, particularly hydrochloric, tartaric, and acetic.

Most investigators agree that at least two advantages are

[1] Koch, R., 1881. Ueber Disinfektion. Mitth. aus dem Kaiserliche Gesundheitsamt, 1, 1–49.

derived from such additions, particularly the addition of the alkali chlorides and the acids, viz.: a lessening of the precipitation of the $HgCl_2$ by the albuminoids present, and greater solubility of the mercuric chloride. A pronounced increase in the toxic properties of these mixtures over that of the pure $HgCl_2$ solution has been claimed by several workers as a third and very important advantage. Laplace,[1] working with anthrax spores, found that hydrochloric and tartaric acids caused a very considerable increase in toxic properties when added to solutions of mercuric chloride of about 1:20,000. He recommends the use of the tartaric acid as being non-volatile and more stable than the hydrochloric. Later Behring,[2] in a very careful piece of work on various antiseptics, found substantial increases in the value of $HgCl_2$ as an agent of this class when certain salts were added to its solution. I copy a few of his results on this point. They were also with anthrax spores.

$HgCl_2$ alone	has a value of			1 : 10,000
$HgCl_2$ + 10NaCl	"	"	"	1 : 15,000
$HgCl_2$ + 3NH_4Cl	"	"	"	1 : 12,000
$HgCl_2$ + 1KCN	"	"	"	1 : 15,000

With Laplace's solution, $HgCl_2$ + tartaric acid, however, he got a somewhat lower value, viz., 1:8,000.

On the other hand, in one of the most recent and at the same time one of the most exact and exhaustive of the studies that have appeared, Krönig and Paul[3] have found that the addition to aqueous solution of $HgCl_2$ of any salt experimented upon had invariably the effect of lessening its toxic value under the conditions with which they worked.

It was with a view of determining the truth in regard to

[1] Laplace, 1887. Saure Sublimat Lösung als desinficirendes Mittel. Deutsche Med. Wochenschrift, 1887, 866–867.

[2] Behring, 1889. Ueber die Bestimmung des antiseptischen Werthes chemischer Präparate mit bes. Berücksichtigung einiger Quecksilbersalze. Deutsche Med. Wochenschrift, 43, 884–887 (1889). See also Central. für Bakteriol, Bd. 1, 27–30 and 64–66 (1888).

[3] Krönig und Paul, 1897. Die chemischen Grundlagen der Lehre von der Giftwirkung und Desinfection. Zeit. f. Hygiene u. Infec. Bd. 25, 1-112.

this matter of toxicology that the present investigation was undertaken. It proved a large task, and at the end of six months of study I am for the present compelled by circumstances to discontinue the work. Inasmuch, however, as I have worked over the addition of the alkali chlorides quite thoroughly and done some satisfactory work on the addition of hydrochloric acid, it was thought well to publish my results at this time.

The work was largely done with the mold fungi. These plants, which have been so generally useful and so generally used in physiological investigations, were found to be especially well adapted for toxicological work, consequently of the more than seven thousand cultures made in this study, about sixty-three hundred were with the molds. The others were with yeasts, bacteria and phanerogams.

The fungi used were : *Aspergillus flavus*, *Sterigmatocystis nigra*, *Œdocephalum albidum*, *Botrytis vulgaris*, *Penicillium glaucum*, *Rhizopus nigracans*, and two yeasts, the species of which were undetermined. Of the phanerogams, I used seeds of wheat, sunflower, pea, lupine, and alfalfa. Of bacteria, *Bacillus colon* (from chick) and an undetermined Bacillus.

Culture Methods. — For a detailed account of my method of using the van Tieghem cell in toxicological work, the reader is referred to my article on " Electrolytic Dissociation and Toxic Effect."[1] This method of culture was used for the molds, yeasts, and bacteria. It will suffice to say here that when the precautions detailed in the above article were observed, especially that requiring the same solution in the bottom of the cell as was used in the culture drop, this method was found entirely satisfactory and reliable, and very well adapted for such studies.

Media. — The bulk of the results recorded here were from cultures grown in a decoction of sugar-beet. The root, sliced thin, in the proportion of 450 grams per liter, was heated in water in an autoclave for an hour at 120° C under a pressure of one atmosphere. The decoction was then filtered, poured into

[1] Clark, J. F. Jour. Phys. Chem. 3, 263–316 (1899).

sterile flasks and again heated at the same temperature for about three hours. This second heating is intended to precipitate as completely as possible the proteid substances present. Immediately before use it was filtered, when cold, through seven filters to remove as completely as possible the precipitated albuminoids.

This medium is excellent for general work with nearly all saprophytic fungi, germination occurring quickly and regularly. Mercuric chloride, however, in any medium admitting of normal development of fungi or bacteria, is subject to serious deterioration from reduction to calomel. If the media be an infusion or decoction of a vegetable or animal substance, a second and even more serious factor causing deterioration is introduced by the presence of a varying quantity of proteid matter in suspension or solution.

The reduction to calomel is a rather *constant* factor, and being the result of the action of light and the presence of organic matter, it may be largely controlled by keeping the solutions of $HgCl_2$ in the dark and at a low temperature. The cultures as soon as prepared were grown in a light-proof thermostat, hence, the media containing $HgCl_2$ were exposed to light only during the preparation of the cultures and their examination from time to time. No media containing $HgCl_2$ was used later than 24 hours after the addition of the $HgCl_2$.

The deterioration due to precipitation by proteid substances was a more serious matter, inasmuch as the amount of proteids present in different batches of media was a variable and often considerable quantity. Long continued heating at 120° C, and the preparation of large quantities at a time were found the most reliable means of overcoming this source of error. The extent of the variation possible from this cause was illustrated by a number of cultures with *Rhizopus* in the pure juice of the beet extracted without boiling, and the same medium after heating for hours at 120° C. In the former case 0.0004 *n* $HgCl_2$ was required to inhibit germination, while in the latter case 0.00001 *n* was sufficient. This indicates that not more than 1/40 of the mercuric

chloride was available for antiseptic purposes in the medium that had not been heated. It was also observed in practice that in working with a medium containing considerable proteid substance, the most of the deterioration took place within a few hours after the addition of the $HgCl_2$. This led to the precaution of adding the $HgCl_2$ to a sufficient quantity of beet decoction for the day's cultures six or eight hours before it was to be used. As already indicated, any solutions remaining unused when the cultures for the day were prepared were discarded. Inasmuch as the beneficial effect of adding compounds to $HgCl_2$ solutions has been attributed to the effect of these compounds in "protecting" the $HgCl_2$ from precipitation by albuminoids, it was thought desirable to use a medium which would be entirely free from such substances, accordingly several series of cultures were grown in the following well-known medium :

Cane Sugar	5 grams
$MgSO_4$	0.25 "
KH_2PO_4	0.5 "
Asparagin	0.5 "
Fe_2Cl_6	trace
Water to	100 cc.

In setting up a series of cultures with $HgCl_2$ to which some salt or acid had been added in varying quantity, checks were usually set up in pure $HgCl_2$ solutions *after* as well as before the cultures containing the compound whose effect was being tested. When the *precautions already mentioned were all observed*, however, these checks usually coincided very closely, showing that the deterioration during the setting up of a complete set — usually about five hours — could be disregarded.

By a complete set of cultures is meant a series of cultures in which the critical point (i. e., the point where germination is inhibited) is determined for the pure $HgCl_2$ solution, and as many other solutions, containing a varying addition to the $HgCl_2$ of the compound whose influence is being tested, as may be required to plot the curve giving the increase or decrease of toxicity, as the case may be, due to the addition of the compound. In my work I

found that twenty-five such solutions covered the field satisfactorily. For example, suppose the effect of adding NaCl to $HgCl_2$ solutions is the problem. The toxic value of the pure $HgCl_2$ being determined, the next step is to determine that of a solution of $1HgCl_2 + 2NaCl$, $1HgCl_2 + 4NaCl$, ..., $1HgCl_2 + 100NaCl$, and so on until a very injurious concentration of the NaCl itself is reached. In the case of *Aspergillus* this would be at approximately 15 percent NaCl, which inhibits germination. Measured in molecules it would mean the addition of about 36,000 molecules of NaCl for each molecule of $HgCl_2$ present. Considering this very great number it might be thought that 25 steps were far too few to cover the ground from + 2NaCl to + 36,000 NaCl, and it certainly would be, had one not in preliminary experiments determined approximately where the important points were. With *Aspergillus*, for instance, and with additions of NaCl (see Fig. 2) it will be noted that the important points are from + 2NaCl to + 1000 NaCl, near + 2000, and near + 15,000 NaCl.

At each of the twenty-five points selected, it was found necessary to make cultures covering a moderately wide range of $HgCl_2$ concentrations, as otherwise the critical concentrations might be missed. From six to twelve such cultures were prepared, the number varying according to the thoroughness with which the preliminary work was done. With *Aspergillus* these cultures should cover a range of from 0.0001 *n* to 0.00002 *n* with the pure checks in $HgCl_2$ and the smaller additions up to + 4000 NaCl, and from 0.00015 *n* to 0.00004 *n* with the stronger concentrations of NaCl. Reckoning ten cultures at each concentration of NaCl, and twenty-five concentrations as the number tested, it would require 250 cultures to develop a curve for a single fungus, as is shown for three in Fig. 2. As a matter of fact we must add at least forty or fifty more to this number for preliminary work.

A stock solution of mercuric chloride in distilled water containing 4.382 percent of the salt was prepared at first in sufficient quantity for the entire study, thus avoiding possible error from

changes. A *normal* solution of $HgCl_2$ means in this paper the number of grams of the salt corresponding to the *full* molecular weight (270) dissolved in a liter of water; thus, 0.01 *n* equals 0.27 percent $HgCl_2$.

Except when otherwise stated, the *point of inhibition* of germination of the spores was taken as the critical point. This was taken in preference to the death-point on account of the greater ease and certainty with which it could be determined, and the fact that in *antiseptic* solutions the inhibiting-point is the point of practical importance. With *disinfecting* solutions, however, the death of the spore is the object, and the concentration at which it occurs the point to be determined. This was done when working with the $HgCl_2$ in disinfecting concentrations. The point of inhibition, however, must be understood to mean the concentration preventing the germination of 95 percent of the spores. The spores normally germinated practically 100 percent, and as the object was to test the effect of adding the salt, etc., on the toxicology of the mercury in solution, it was obviously safer to read the results from the intermediate 90 percent, disregarding the 5 percent most easily killed as well as the 5 percent abnormally resistant.

In many cases the spores from inhibited cultures were transferred to pure beet infusion and their vitality thus tested. In such transfers more or less of the deleterious agent is necessarily transferred with the spores. The general results of such transfers, however, indicated that with fungus spores a solution which inhibited germination would eventually kill if left in contact with the spores a sufficient length of time. Thus if a solution of a certain concentration just inhibited germination, a solution of four times this concentration might with some spores fail to kill in 24 hours; if left for ten days, however, the points of inhibition and death would usually nearly coincide, and with many forms this would be true in a much shorter time.

The temperature at which an antiseptic or disinfectant is applied has been shown to be very important.[1] The effectiveness

[1] Henle, 1889. Ueber Creolin und seine wirksamen Bestandteile. Arch. f. Hyg. Bd. 9, Heft 2.

of the agent, other things being equal, increases with the temperature. In a number of experiments with *Aspergillus* I concluded that its greatest resistance to mercuric chloride was at a temperature near (apparently slightly below) the optimum for the germination and development of the fungus. Cultures above this point were killed because of the greater effect of the agent at the higher temperature. Cultures below this point were killed by the penetration of the poison before the spores had succeeded in the production of any considerable amount of proteid matter with which to neutralize the poison, thus cultures kept at 16° C were killed by a very small amount of $HgCl_2$, germination being so greatly delayed by the low temperature, time was given for the agent to have its maximum effect. In general, then, mercuric chloride may be said to be more effective either below or above the optimum temperature for the development of the organism or organisms to be checked; the lower temperature requiring the longer contact with the deleterious agent, the higher being effective in a shorter time. This is probably true of all antiseptics which, like mercuric chloride, check or destroy by precipitation of the contents of the living cell. It *may* be true of all toxic agents. The temperature at which my work was done was 28° C, a very favorable temperature for the development of the fungi with which I was working.

Sterile precautions were observed throughout the study. The spores used in the inoculation of the cultures were taken from pure cultures frequently renewed to insure high vitality. It is very important in any quantitative study of this kind to have as nearly uniform a number of spores in the various cultures to be compared as possible. It is equally important to avoid bunches of spores in making the inoculation. Oversight in regard to these two points leads to the most erratic results in working with a compound like $HgCl_2$, which in effecting its mission as a disinfectant, is itself precipitated in a harmless insoluble form. A moment's reflection will show how this is necessarily so. The amount of $HgCl_2$ required to kill say two hundred spores in a few hours may fail to kill ten out of a thousand in a medium where

growth is possible before the poison has had time to have its effect. So, too, if a bunch of spores be introduced into a culture containing many single ones, the medium is relatively slow in reaching those bunched, especially when such a bunch contains air, as it almost invariably does. The result is that those floating singly are first killed, and the solution may be depleted of half or more of its toxic value before those enclosed in the bunch are reached. These then may germinate and grow almost as well as if no poison had been present. The difficulty was entirely overcome by soaking the spores for a longer or shorter time in sterile distilled water, with an occasional shaking. In some cases the soaking may be continued with advantage for days, in which case it is well to keep them at a low temperature to prevent germination. In all cases it was found advisable to inoculate from a mixture of spores in water, a uniform inoculation being approximated in that way without great difficulty.

The method of preparing the solutions of various concentrations of the added compound was briefly as follows: (to illustrate, I shall assume that *Aspergillus* is the form to be tested.) to a liter of beet decoction is added sufficient $HgCl_2$ to make 0.00015 *n*. To a portion of this enough NaCl (dry) is added to make the strongest concentration of NaCl desired. With these two solutions as a base any intermediate concentration may easily be prepared by using carefully graduated pipettes.

This method implies an error in the concentration of the $HgCl_2$ present, inasmuch as the *dry salt added* dilutes the $HgCl_2$ solution somewhat. This error, however, is *constant*, *easily calculated*, and *practically nil* until the concentration of the added salt reaches far into the thousands. At + 2000 NaCl it would be less than 0.3 percent of the total toxic value.

In preparing my results for publication, I have endeavored to present them in the briefest possible space. To this end I have decided to plot the general results in curves, giving as they do the significant facts without obscuring them with a mass of detail. The data have been further condensed by publishing only typical curves of the various classes. Thus, for example,

while curves were worked out for seven fungi with sodium chloride, and five each for potassium and ammonium chlorides, only three of those for sodium chloride are herewith published, and none of those with either potassium or ammonium chloride, because they were practically the same as those with NaCl, and while invaluable in confirming that work, threw no new light on the problems involved. Complete sets were also run through with HCl with five fungi, but the results with *Sterigmatocystis* given in Fig. 5 give as much light on the subject as any other of the five or all the five, hence, only the one curve is given.

To give the reader an idea of the data from which these curves were developed, I shall insert here as Fig. 1 a page from my note-book, showing a short-checking series with *Aspergillus* in the non-albuminoid medium. On the left are the concentrations of $HgCl_2$ for the various cultures. Then arranged in order of preparation are a series of 11 sets, the last one being like the first, a set with $HgCl_2$ alone to test the deterioration of the solutions while the cultures were being prepared. The cultures are lettered and numbered, the letters referring to each set of twelve, the numbers to the cultures of that set, No. 12 being always the most concentrated. In order to prevent confusion in this limited space, I insert only the final estimate of germination in each culture. Cultures from *e* to *j* were much slower in germination than those containing but little salt. It will be noticed that 0.000025 *n* was the limiting culture with *Aspergillus* here. This was higher than was usual in a beet decoction, where the average was about 0.000075 *n* because of the albuminoids present. One batch of beet decoction, however, gave a value of practically the same as this, showing it to be almost free from proteid matter. It should be added that no curve was plotted without a repetition of the critical cultures on which it was based at some time other than when the entire series was set up. This method was adopted in place of duplicate cultures because it implied a checking in the computations and measurings as well as the observed germination.

Figure 2 gives the curves for three fungi, showing the toxic

Aspergillus flavus. Cultured December 31, 1900. Temperature 28° C.

Concentration of $HgCl_2$	No. of culture	+ 0 NaCl *a*	+ 10 NaCl *b*	+ 40 NaCl *c*	+ 400 NaCl *d*	+ 1000 NaCl *e*	+ 4000 NaCl *f*	+ 7000 NaCl *g*	+ 10,000 NaCl *h*	+ 15,000 NaCl *i*	+ 20,000 NaCl *j*	+ 0 NaCl *k*
.000008	1	normal	normal	normal	normal	40	40	normal	normal	normal	normal	normal
.000011	2	"	"	"	70	6	15	"	"	"	"	"
.000014	3	"	"	8	5	1	4	"	"	"	"	"
.000017	4	80	65	4	3	0	1	"	"	"	"	"
.000020	5	30	5	0	1	0	?	90	"	"	"	40
.000025	6	5	2	1	0	..	1	70	"	"	"	8
.000030	7	0	0	0	0	..	..	5	85	"	"	1
.000035	8	0	0	0	..	..	..	3	80	"	"	0
.000042	9	..	..	..	..	..	..	..	10	90	90	0
.000051	10	..	..	..	..	..	..	..	1	60	70	..
.000071	11	..	.	..	..	..	..	..	..	30	50	..
.000100	12	..	..	..	..	..	..	..	..	25	7	..

Fig. 1

values of the $HgCl_2$ solutions to which NaCl has been added. The ordinates give the concentration of the $HgCl_2$ in fractions of a normal solution, thus *Aspergillus* is inhibited by 0.000075 *n*.

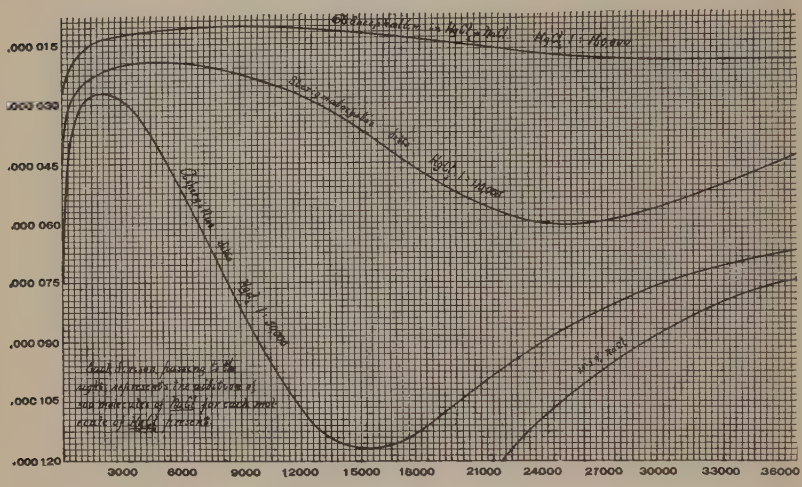

Fig. 2

The abscissæ give the additions of NaCl in molecules. Thus 3000 NaCl indicates a concentration of 3000 molecules of NaCl for each molecule of $HgCl_2$ present.

A very striking feature of this figure is the very rapid rise of the curves with the smaller additions of the NaCl. This is followed by a less rapid but equally marked fall with all forms. It is also to be noted that the maximum is reached with a relatively larger number of added NaCl molecules, as the fungi are more sensitive to $HgCl_2$. Thus, while *Aspergillus* reaches a maximum with about + 2000 NaCl, *Œdocephalum* reaches its maximum at about + 9000 NaCl. The actual percentage concentration, however, is not nearly so different on account of the fact that with *Aspergillus* it is + 2000 NaCl at a $HgCl_2$ concentration of about 1 : 200,000, while with *Œdocephalum* it is + 9000 NaCl at a $HgCl_2$ concentration of little more than 1 : 400,000. It is a significant fact, however, that the more dilute the $HgCl_2$ solution, the higher the percentage addition of NaCl

required to give either an increase or decrease in the toxic value of the mixture. A glance at the falling of the curves will also show this. The upper curves fall neither so fast nor so far as that of *Aspergillus* in the more concentrated $HgCl_2$ solutions. The second rise in the lower two curves is due to the added NaCl, which is here present in injurious concentration. The short curve in the lower right hand corner indicates a 15.2 per cent concentration of NaCl, which is sufficient alone to inhibit *Aspergillus* and *Sterigmatocystis*. Nine percent NaCl inhibits *Œdocephalum*. These curves then represent the combined inhibiting influence of the $HgCl_2$ and the NaCl present. In the more dilute concentrations of NaCl, say up to + 4000 NaCl, the influence of the NaCl as such is almost or altogether nil. With + 15,000 with *Aspergillus*, and + 25,000 with *Sterigmatocystis* it is appreciable.

In Fig. 3 the curve is that of *Aspergillus* in solutions of $HgCl_2$ + NaCl, but the theoretical value of the NaCl is subtracted. 30,000 molecules of NaCl are equal to 1 molecule of $HgCl_2$ as an inhibiting agent for *Aspergillus*. Theoretically, therefore, if 1000 molecules of NaCl be added for each molecule of $HgCl_2$ present in a certain solution the toxic value of that solution would be increased 1/30 of its original value. I say *theoretically*, for it is doubtful if that addition of NaCl would *as such* have any toxic value. To be on the safe side, however, the exact proportion of the toxic value for each addition of NaCl has been deducted in plotting this curve. Consequently the curve gives the toxic value of the *mercuric chloride as influenced by the presence of the NaCl.*

In this figure the ordinates represent units of toxicity. The value of mercuric chloride in the absence of NaCl is represented by unity.

The abscissæ represent the amount of NaCl added in fraction of normal solution and as a percentage.

The rise of the curve with additions of NaCl in small amounts up to 0.4 percent is very striking. At about 0.4 percent NaCl a maximum of about 160 percent increase is reached

further additions presently cause a fall which reaches unity again at about 2.5 percent of NaCl. From this onward the decline is steady until at a 10 percent concentration of NaCl the mercury has not more than one-fifth its normal value when in the form of the bichloride.

It must always be borne in mind that this is for a fungus having a critical concentration for the pure $HgCl_2$ solution of about 1:50,000. As already noticed, and emphasized later, en-

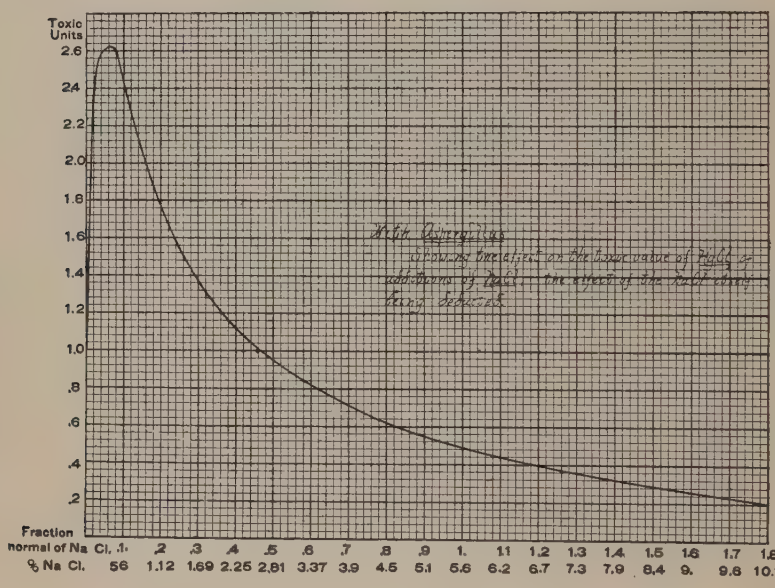

Fig. 3

tirely different conditions obtain for solutions of greatly varying concentrations.

After getting these results with beet decoction as a medium, the cultures were repeated, using the artificial medium compounded from salts and sugar already given. An entirely similar curve was gotten for *Aspergillus* and *Sterigmatocystis*, thus giving an emphatic negative to the theory so frequently advanced by bacteriologists that the value of the NaCl, used in connection with $HgCl_2$, has some relation to its "protective"

powers in protecting the $HgCl_2$ from precipitation by the proteids present. Here there was of course no proteid. It is of interest to note in this connection that the presence of asparagin does not reduce the toxic value of $HgCl_2$. With copper sulphate and other copper compounds I have found it to cause a very striking decrease when present either in pure water solution or in connection with nutrient media.

While Figs. 2 and 3 show a very remarkable increase in toxic properties of the $HgCl_2$ solutions on the addition of small quantities of NaCl, Fig. 4 will show that this increase, while appreciable from the first, is by no means so sudden as might have been thought from Figs. 2 and 3. It is true that the first ten or twenty molecules added give a greater net increase than any ten or twenty added later, but this greater influence of the early additions is not such as to indicate any relation to the effect

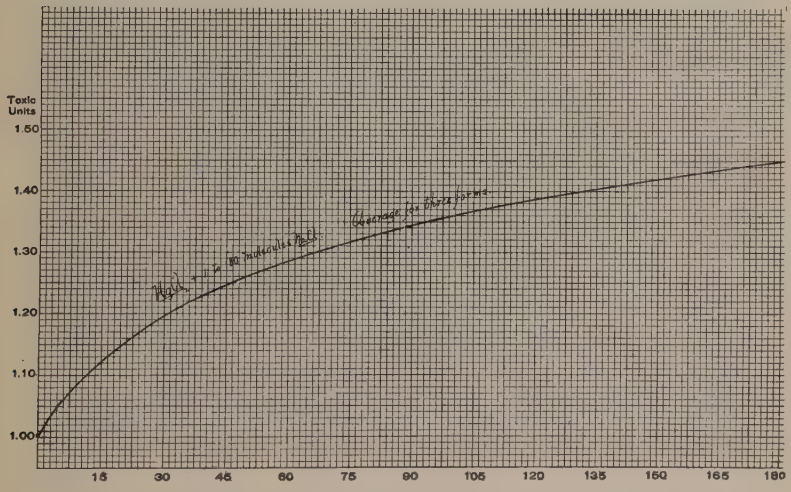

Fig. 4

Abscissæ here represent the number of NaCl molecules added for each molecule of $HgCl_2$ present

of the NaCl on the dissociation of the $HgCl_2$ as such. We will return to this point later.

A very marked stimulation of mycelial development and

some stimulation of germination was noted with *Sterigmatocystis* and *Œdocephalum* with the smaller additions of NaCl in cultures too dilute to be inhibited. This stimulation reached its maximum between + 5 and + 10 NaCl.

Figure 5 gives the result of adding varying quantities of HCl to $HgCl_2$ solutions. The second curve is inserted for comparison and gives the result of adding NaCl in similar proportions to similar solutions of $HgCl_2$ with the same fungus, *Sterigmatocystis*. The influence of the HCl and NaCl as such has been subtracted as in Fig. 3; hence the curves here indicate the toxic value of the mercury conbination only, when in the presence of the varying concentrations of hydrochloric acid and its sodium salt.

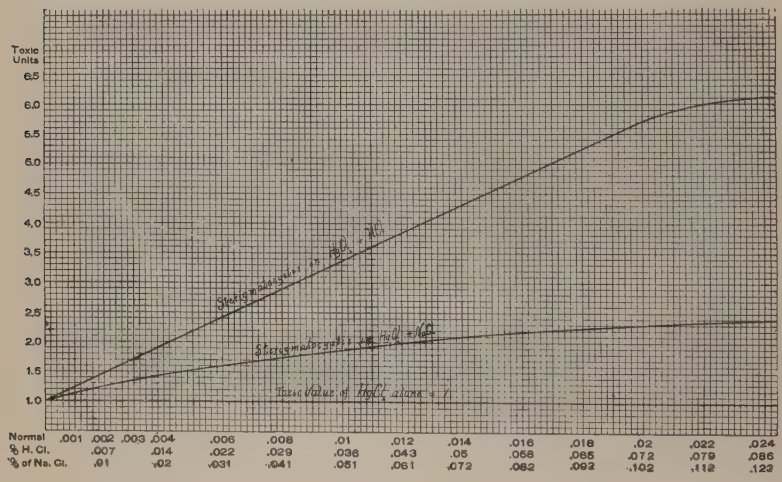

Fig. 5

The upper curve, which is a straight line for a considerable distance, shows a very great increase in toxic properties, amounting to about 500 percent with the addition of HCl in a concentration of 0.022 *n*, or less than 0.08 percent. *Aspergillus*, *Œdocephalum* and *Rhizopus* gave similar results, but their percentage increase was somewhat less than that of *Sterigmatocystis*. This

is rather remarkable, inasmuch as *Sterigmatocystis* is much more *resistant* than pure HCl, and therefore to ionic $\overset{+}{H}$ than any of the other forms.[1]

The additions of HCl were not carried far enough to determine whether there would eventually be a decrease in the toxic value of the mercury compound or not. The toxic value remained practically constant from + 1100 HCl to + 3000 HCl (0.08–0.21 percent). The fact that the curve is at first a straight line indicates that within limits the increase in toxic properties is in direct proportion to the amount of acid added. It has already been noted that with the salts the first additions are always relatively more effective than the same quantity added later, thus giving a distinctly convex curve.

Thus far we have treated of solutions of $HgCl_2$ of no greater concentration than 1:50,000. Some tests were made to determine the effect of adding NaCl to *disinfecting* as opposed to *antiseptic* solutions. The method adopted was to shake up spores in comparatively concentrated solutions of $HgCl_2$ for a short time, then, after removing the $HgCl_2$ solution by filtering and washing, to germinate the spores in a favorable medium.

Rhizopus nigracans was selected for this study on account of the size of the spores, the ease with which they were evenly distributed throughout the solution, and their very dark color, enabling one to recognize them easily on the filter. The critical concentration for this form shaken up with $HgCl_2$ solutions for 75 seconds proved to be about 1:3700. The removal of the $HgCl_2$ from the spores before testing their vitality is the most difficult and important point. My method was to prepare a filtering cone having four times as many perforations on one side as on the other. The single ply of the folded filter was placed next this side. The filter was first wet with water and then connected with an air-pump. When the $HgCl_2$ solution was poured in, the fluid was almost instantly removed, leaving most of the

[1] Clark, J. F. On the toxic effect of deleterious agents on the germination and development of certain filamentous fungi. Bot. Gaz. 28, 289 (1899).

spores evenly distributed over the single ply of the filter. A stream of water was then turned on and the washing continued until free from $HgCl_2$. This was tested by allowing the moist filters to lie for a few hours, and then by compression of those through which the most concentrated solutions were filtered a few drops of water were obtained. To this was added some beet decoction and a few fresh spores. The germination of these spores was accepted as evidence of thorough washing. In taking the washed spores from the filter, care was taken to test those in the most favorable position for thorough washing.

The results of these tests are plotted as curve No. 3 (from

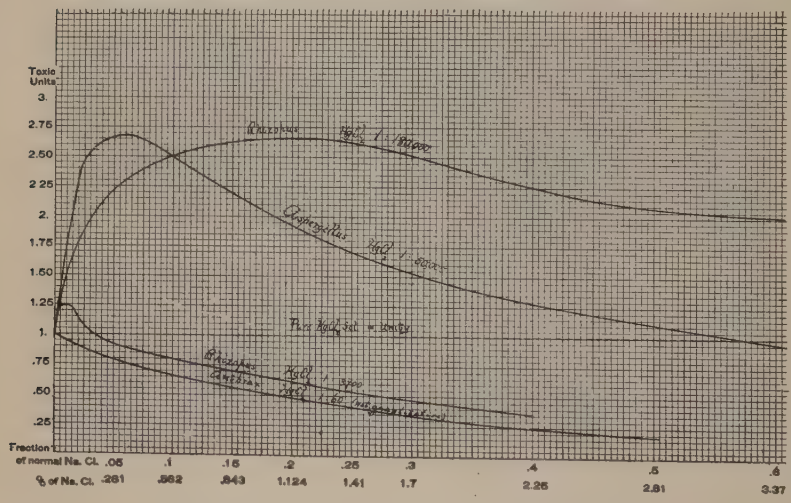

Fig. 6

the top) in Fig. 6. The upper curve is derived from notes on *Rhizopus* grown in beet decoction, concentration of $HgCl_2$ 1 : 180,000. The second curve is the first part of the *Aspergillus* curve plotted in Fig. 2, $HgCl_2$ concentration being 1 : 50,000. The characteristic variation due to variation in concentration already remarked in discussing Fig. 2 is here very well brought out. The third curve is with the same fungus as the first, the difference being due to the greater concentration of the $HgCl_2$ in the

former case. The increase in toxic properties resulting from additions of NaCl was only slight, and due to the adding of very small quantities of the salt. In a concentration of $HgCl_2$ of 1:180,000 *Rhizopus* shows a continued increase in toxic value for the solution on the addition of NaCl up to over 1 percent. When the $HgCl_2$ is present in 1:3700, however, the same plant shows that the mixture is already worth *less* as a toxic agent when but 0.2 percent NaCl has been added than the original pure $HgCl_2$ solution. The fourth curve here is from Krönig and Paul's work.[1] The data at my disposal do not enable me to draw a strictly quantitative curve, but it is drawn in such a way as to show two facts very clearly shown in their tables, viz., that there is a *lowering* of the toxic value of the mixture when any NaCl is added, and that the lowering is relatively greater with additions up to 1.5 percent than it is from that point to 3.3 percent NaCl. The smallest addition of NaCl they experimented with was 0.4 percent, and their concentration of $HgCl_2$ was 1:60. Their results, then, are in fullest accord with the general facts shown in the three higher curves. The four curves given here will, perhaps, throw a great deal of light on the apparently contradictory results of other workers. It will be remembered that those who found an increase in toxic properties on the addition of NaCl worked with the more dilute solutions of $HgCl_2$, while Krönig and Paul who found the contrary worked with very concentrated solutions.

A careful study of Fig. 6 shows that whether or not an increase or decrease is to be anticipated from an addition of NaCl to $HgCl_2$ solutions, depends altogether on two considerations:

(1) the concentration of the $HgCl_2$ present and

(2) the amount of the NaCl or other chloride added. A much higher percent of the salt must be added, however, if we would get a *decrease* of the toxic properties with the more dilute solutions of $HgCl_2$.

Fig. 7 illustrates a further peculiarity of concentrated $HgCl_2$ solutions to which NaCl has been added in considerable

[1] Krönig and Paul. l. c., p. 49, Tables 26 and 28.

concentration. Here the abscissæ represent the concentration of $HgCl_2$ + a definite number of NaCl molecules. In the upper part of the figure it is $1HgCl_2$ + 100NaCl, in the lower part $1HgCl_2$ + 10NaCl. The ordinates represent the development of the fungus and the bacterium in the various concentrations. The two upper curves are from my notes on *Rhizopus* and are taken from the same series as curve No. 3 in Fig. 6, the ordinates representing the percent germinated. The lower are from Krönig and Paul's[1] Table 28, and the development of the cultures is

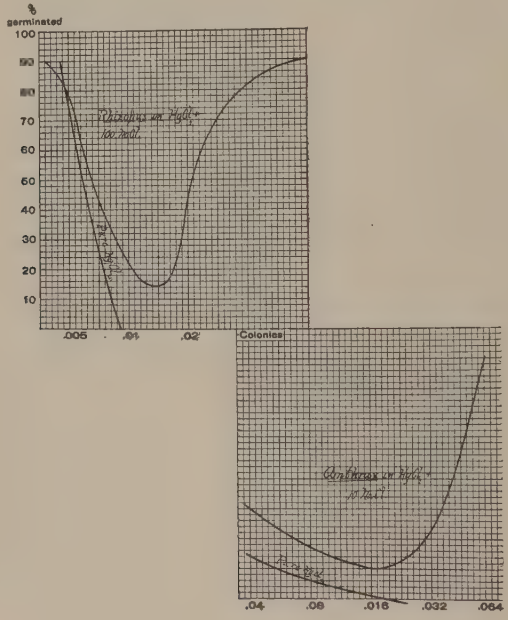

Fig. 7

Abscissæ represent the concentration of $HgCl_2$ in fractions of normal solution. The ordinates in the upper part give the percent of *Rhizopus* germineted, in the lower part the number of colonies of anthrax developed.

represented on the ordinates by the number of spores of anthrax that survived immersion in the disinfecting solution for twenty minutes. The upper of the two curves represents in each case the $HgCl_2$ + the NaCl. The lower of each set represents the

[1] Krönig and Paul. l. c., p. 50.

effect of a pure $HgCl_2$ solution. The upper curves show that except in the very dilute solutions the pure $HgCl_2$ solution is the more toxic. The curves descend together with increasing concentration until about 0.8 percent is reached, when the $HgCl_2$ + NaCl curve takes a sudden and very marked curve upward until it reaches a point indicating an extremely low toxic value where the NaCl is present in about 6 percent concentration. The lower curves are developed in a similar way. Here the $HgCl_2$ is present in about ten times the concentration used with *Rhizopus* because of the greater resistance of *Anthrax*, and the NaCl being ten to one of the mercury, instead of a hundred to one as before, is present in approximately the same percentage concentration in both cases. Here the $HgCl_2$ curve bends consistently downward with each increase of concentration of $HgCl_2$. The same is true of the $HgCl_2$ + NaCl curve — although the mixture is uniformly less toxic — until it reaches a concentration of between 0.8 and 0.9 percent NaCl, when, like its mate above, it curves upward in an extraordinary manner.

Here we have a solution of 1.56 percent $HgCl_2$ + 9 percent NaCl, which may be increased markedly in toxic properties by simple dilution with pure water to four times its volume. The result with *Rhizopus* is entirely similar in kind, although it took place at a much greater dilution of the $HgCl_2$. That a toxic mixture may be made more toxic by simple dilution with water was so startling to me that the result was only accepted after a careful repetition of the work. With this in view, all available literature on the toxicology of $HgCl_2$ was reviewed to see if anything similar had been observed by other workers. The above example from Krönig and Paul was the only one found, and it seems to have been passed unnoticed by these workers, although it is quite clearly shown by their table No. 28.

The work with phanerogams was done by preparing 400 cc solutions of $HgCl_2$ of various concentrations and similar solutions of $HgCl_2$ + various additions of NaCl. The seeds, which were carefully selected, were put into these solutions in the dry state, and after soaking forty-eight hours at about 23° C, were

removed, washed well in water, and germinated in sphagnum moss. I am not satisfied that this method is above criticism, and prefer not to say more than that the resistance of the phanerogams to $HgCl_2$ is in general very similar to that of the fungi, and that my experiments indicated clearly a similar relation to $HgCl_2$ solutions containing NaCl as that worked out for the fungi. The legumes, however, while showing an increase in toxic properties with the smaller additions of NaCl, apparently did not show any falling of the curve with increasing concentration of NaCl. More work, however, must de done on this point.

The yeasts and bacteria showed a remarkable difference in their resistance to $HgCl_2$. The two yeasts used were the most resistant of all plants tested, while the bacteria are, at least in the vegetative condition, extremely sensitive to very minute quantities of this agent. My cultures with the yeasts in $HgCl_2$ + NaCl solutions were somewhat erratic from some undetermined cause, and I propose to do further work before publishing. Few cultures were made with bacteria in solutiens to which NaCl was added, those which were made, however, showed that *Bacillus colon* was influenced by additions of NaCl in a way entirely similar to the fungi.

In concluding this review of my experimental data I wish to say that *Botrytis vulgaris* seems to be an exception to the rule established by the other molds. It is by far the most sensitive to $HgCl_2$ of the fungi tested. It is inhibited by about one-eighth the concentration required to inhibit *Œdocephalum*, itself a very sensitive form. *Botrytis* was tested nine times to test the effect of adding chlorides and hydrochloric acid to solutions of $HgCl_2$. And in no case, except possibly with HCl, did it show any evidence of increased toxic properties in the solution until the added compound was itself present in deleterious concentration.

In discussing the results of this work, it may not be out of place for even a botanist to express his opinion of the chemical significance of the facts observed. Krönig and Paul in summing

up their work on mercuric chloride, especially their results with adding chlorides to the solutions, concluded that the lowering of the toxic value by such additions was due to the suppression of the dissociation of the $HgCl_2$. Were this view correct the additions of chlorides to more dilute solutions would result in a still more marked decrease in toxic value, inasmuch as the $HgCl_2$ would with greater dilution be more fully dissociated. We have found the contrary to be the case. Further they overlooked the fact that the $HgCl_2$ in the concentrations used by them (mostly 1.69 percent) would certainly not be appreciably dissociated. They explained the observation that the cadmium chloride reduced the toxic properties less than other chlorides on the basis of its less dissociation; hence its lessened ability to reduce the dissociation of $HgCl_2$. In view of what is now known regarding the toxicology of cadmium[1] the correct interpretation is doubtless to be found in the toxic effect of the cadmium itself.

It is established that the addition of chlorides to solutions of $HgCl_2$ results in the formation of double salts in which the $HgCl_2$ forms a part of a more complex molecule. A number of such double salts have been isolated in crystalline form. My interpretation of my results and the results of other workers is based on this fact, and on the probability that these complex salts dissociate readily, or at least comparatively readily into their ions. To illustrate this, we shall consider the case of NaCl only added to $HgCl_2$ solutions, and of the possible double salts only the well known Na_2HgCl_4 will be considered in detail. What is true of the formation and dissociation of this double salt is doubtless true with minor variations of all the double salts of the alkali chlorides. As already mentioned, quite a number of such salts have been isolated, and it may be that many occur in solutions with varying concentrations of the alkali chloride or the $HgCl_2$ which have not yet and perhaps cannot be isolated without decomposition.

When NaCl is added to $HgCl_2$ even in great dilution, we

[1] Clark. J. F. Bot. Gaz. **28**, p. 389.

must suppose that more or less of the double salt results. On its dissociation we would get two $\overset{+}{Na}$ and one $\overset{-}{HgCl_4}$ ion. The addition of more NaCl within certain limits would increase the concentration of $\overset{-}{HgCl_4}$ ions. That the replacing of the $HgCl_2$ molecule by the $\overset{-}{HgCl_4}$ ions is likely to result in an increase of toxic properties, is, it seems to me, not only probable, but almost certain, being in harmony with all our knowledge of toxic agents which have the power of combining with chlorine in varying proportions. To quote from Davenport,[1] "Beginning with methane, CH_4, we find this substance — marsh gas — inocuous when mingled with air. As the H atoms become replaced by one or more chlorine atoms, the poisonous properties increase.

CH_3Cl is slightly anesthetic.
$CHCl_3$ = chloroform.
CCl_4 is very dangerous, stupefying involuntary muscles."

This relation of chlorine to toxicology is quite well established, and is in fullest harmony with the upward curves of all my figures, when the solutions are dilute and the added chloride is not present in excess. With an increasing concentration of NaCl, however, there must come a time when the double salts can no longer readily dissociate on account of the high concentration of $\overset{+}{Na}$ ions present from the great excess of NaCl, which is not in combination with the $HgCl_2$, and also from the increased concentration of the $\overset{-}{HgCl_4}$ ions. This continued increase of the NaCl would result then in three changes in the physical condition of the dissolved salts.

1. A lessening of the $HgCl_2$ molecules present, if all were not already combined to form the double salts.

2. A lessening of the $\overset{-}{HgCl_4}$ ions.

3. An increase in the number of complex molecules containing mercury, e. g. Na_2HgCl_4.

[1] Davenport, C. B., '97. Experimental Morphology, Part I.

Should these large and complex molecules — and it may be that in high concentrations they are very large and complex — have a lower toxic value than the pure $HgCl_2$ molecule, we have an explanation for the radical change occurring in the curve, indicating that the additions of NaCl no longer increase, but markedly decrease the toxic value of the mixture.

In this connection it is highly significant, as has already been hinted, that in all cases tested the concentration of the $HgCl_2$ at the critical points was the determining factor as to whether an increase or decrease of toxic value was to be expected from any given addition of a chloride. This is true alike for antiseptic and disinfecting concentrations. In the latter case we should expect that the greater concentration of $HgCl_2$ would facilitate the formation of the double salts, and the formation of these salts would presently prevent their own dissociation in the relatively concentrated solution. Fig. 6, Curve No. 3, shows that with an addition of only 0.08 percent NaCl the toxic properties of the $HgCl_2$ solution are being already reduced. It must be noted that this is but *one-fifteenth* the concentration of NaCl required to cause a falling of the curve with the same fungus but in a very much greater dilution of the $HgCl_2$. (Compare curves 1 and 3, Fig. 6.)

Finally Krönig and Paul, working with very concentrated solutions, found that salt added to such in 0.4 percent or greater concentration invariably lowered the toxic value. Here we must suppose, according to our theory, that the concentration of the $HgCl_2$ is too great to admit of the dissociation of the double salts formed.

I regard the results given in Fig. 7 as further evidence in favor of the view that in the molecular form these complex molecules are of a lower toxic value than $HgCl_2$, but of higher value when ionized. Here with different forms, different methods, different concentrations, and different workers, it is shown that solutions of $HgCl_2 + NaCl$ when the salts — particularly the NaCl — are present in great concentration, *a great increase in the toxic properties of the mixture may be obtained*

by the simple diluting with water to four or more volumes. It would appear that the most reasonable explanation is that the greater dilution enabled the double salts to dissociate, thereby giving us highly toxic $\bar{H}g\bar{C}l_4$ ions instead of the comparatively harmless Na_2HgCl_4 molecule.

Just why *Botrytis vulgaris* proved an exception to the rule established by the other molds is unknown. There is, however, undoubtedly such a factor in biology as *specific resistances* of the protoplasm of certain organisms to certain agents. *Botrytis* itself has such a specific resistance to $AgNO_3$ as compared with *Penicillium*.[1] One-eighth the concentration of mercuric chloride required to kill *Penicillium* is fatal to *Botrytis*. With silver nitrate the exact converse is the case four times the concentration fatal to *Penicillium* is required to inhibit *Botrytis*. I offer it merely as a suggestion that *Botrytis* may have a specific resistance to the complex $\bar{H}g\bar{C}l_4$ ion.

Just why HCl should be so much more effective than NaCl (see Fig. 5) in increasing the toxic properties of $HgCl_2$ solutions is also hard to understand. It does not appear, *a priori*, that we have any reason to suppose that it unites more readily with $HgCl_2$ than its sodium salt, or being united, that the resulting complex molecule would dissociate much more readily, as would be required were we to account for the increased toxicity of the mixture in the same way as we have done with the sodium salt. It may be that here we have an example of increased toxicity due to the catalytic action of the $\dot{H}$ ions on the very toxic ion, $\bar{H}g\bar{C}l_4$.

Whatever the chemical or physical conditions in these mixtures may be, it is demonstrated beyond doubt, that the recommendations of additions of NaCl to solutions of mercuric chloride for disinfecting purposes in practical sanitation have been without sufficient experimental basis. Every formula recommending the additions of NaCl for this purpose which has come to my notice has prescribed NaCl in a proportion that is

[1] See my Bot. Gaz. article, p. 311.

undoubtedly a very considerable detriment to the usefulness of the disinfectant.

The fact that it renders the $HgCl_2$ more soluble, and the claim that it is not so readily precipitated by albuminoids will not offset this loss of toxic properties.

Mercuric chloride is *readily soluble in hot water*, and the cold saturated solution retains about 5 percent of the salt in solution, a concentration vastly stronger than is required for any sanitary purpose. The claim that in the presence of NaCl the $HgCl_2$ is not so readily precipitated by proteids in solution is, in view of all work done on the subject, probably entirely correct. I would emphasize, however, that this is but another way of saying that the double salts of $HgCl_2$ + NaCl *have less affinity for proteid substances than the pure* $HgCl_2$ *solution.* Let us see where this leads us. The whole value of mercuric chloride as a toxic agent whether as an antiseptic or as a disinfectant, is due to this very remarkable affinity for proteid matters. It kills the living cell by chemical union with proteids necessary for the continuance of life. In this chemical union the $HgCl_2$ is necessarily precipitated as well as the proteid of the cell in the form of a quite insoluble mercury albuminate. Now it is patent that any treatment that "protects" $HgCl_2$ from being precipitated by the proteids must conversely protect proteids from precipitation by the $HgCl_2$, and must therefore be a positive and very serious detriment to the carrying on of the work of disinfection.

In the use of mercuric chloride as a fixing agent for tissues in biological studies, we find another case where an apparent advantage has been accepted as a real one without sufficient experimental work. It is the custom in many laboratories to recommend that the $HgCl_2$ for fixing purposes be dissolved in a "normal" salt solution, by which is meant a 0.6 percent solution of NaCl. Ten percent of $HgCl_2$ is easily dissolved in such a salt solution and the presumption is that a solution containing 10 percent $HgCl_2$ would necessarily be a much more rapid fixer than a saturated solution of the pure $HgCl_2$ where but 5 percent of the $HgCl_2$ is in solution. The probability is that there is a

sufficient toxic power in either of these solutions to cause almost instant fixation, but there can be no doubt but that the pure $HgCl_2$ solution is the better in regard to toxic properties. And I have not known anyone who has used both who has had any other reason than the *amount of $HgCl_2$ present* for believing the contrary.

In conclusion I beg to acknowledge my indebtedness to Dr. B. M. Duggar, professor of plant physiology, and to Professor Geo. F. Atkinson, professor of botany in Cornell University, for every courtesy and assistance in the prosecution of this study. My best thanks are also due to Professor W. D. Bancroft for invaluable suggestions on the chemical aspects of the work.

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ON THE TOXIC PROPERTIES OF SOME COPPER COMPOUNDS WITH SPECIAL REFERENCE TO BORDEAUX MIXTURE.

JUDSON F. CLARK.

(WITH SEVEN FIGURES)

WITHIN recent years copper has come to be the most valued agent in combating the ravages of plant diseases. Its importance from an economic standpoint has attracted to its study an unusually large number of investigators, and as a result there is a very extensive literature on the toxicology of copper compounds. The object of the present study was to determine more exactly the concentration of the various copper compounds required to inhibit germination or to kill the spores of a fairly large and quite representative selection of fungi grown under favorable conditions; to determine the effect of various media on the toxicology of copper; and to throw light on the toxicology of the Bordeaux mixture. I shall not attempt to review the literature of the subject, except to refer very briefly from time to time to a few studies which are of special interest in connection with points in the present study. Those especially interested will find work before 1896 quite carefully reviewed by Fairchild,[1] Swingle,[2] and Evans.[3] References and reviews of work since that time may be found in the various reviews. Hollrung's Jahresbericht[4] is especially useful in this connection.

[1] FAIRCHILD, D. G.: Bordeaux mixture as a fungicide. Bull. no. 6. Div. Veg. Path. U. S. Dept. Agric. 1894.

[2] SWINGLE, WALTER T.: Bordeaux mixture, its chemistry, physical properties, and toxic effects on fungi and algae. Bull. no. 9. Div. Veg. Phys. and Path. U. S. Dept. Agric. 1896.

[3] EVANS, WALTER H.: Copper sulphate and germination. Bull. no. 10. Div. Veg. Phys. and Path. U. S. Dept. Agric. 1896.

[4] HOLLRUNG, M.: Jahresbericht über die Neuerungen und Leistungen auf dem Gebiete des Pflanzenschutzes. Berlin 1898 and 1899.

Copper occupies an intermediate position in regard to toxic value for the mold fungi.[5] It is surpassed more than a hundredfold by mercuric chlorid, silver nitrate, and potassium chromate and bichromate. It is a little less toxic than nickel and a little more so than cobalt; distinctly more toxic than iron and the mineral acids; twelve times as toxic as zinc; and about eighty times as toxic as ethyl alcohol. Its great superiority as a fungicide lies in its cheapness, the tenacity with which its hydroxid, when properly prepared, adheres to the foliage of plants, and, as I shall show later, the solubility of its hydroxid in the presence of or by means of substances found alike in the cell sap of the host plant and of the parasite, but more especially the latter.

For a detailed description of the method of using the Van Tieghem cell for work of this kind the reader is referred to my article in a former issue of this journal.[6] I may add that the method has proved eminently satisfactory for various physiological and toxicological studies with pure cultures of fungi, yeasts, and bacteria.

Precautions for sterilizing were observed throughout the study, except with a few cultures of hymenomycetes. The spores used, of such fungi as can be normally matured in artificial culture, were taken from pure cultures frequently renewed to insure high vitality. Rhizoctonia and currant cane blight cultures were inoculated with mycelium taken from fresh pure cultures. With Hypholoma and Coprinus,[7] spores taken from plants growing under natural conditions were used. Naturally, every precaution was taken to avoid contamination, and on the whole very little difficulty was experienced on this account.

It is very important in any quantitative study of this kind to

[5] CLARK, J. F.: On the toxic effect of deleterious agents on the germination and development of certain filamentous fungi. Bot. Gaz. **28**: 289–327, 378–404. 1900.

[6] CLARK, J. F.: Bot. Gaz. **28**: 293. 1900.

[7] I beg to acknowledge my indebtedness to Dr. Margaret C. Ferguson, of Cornell University, who supplied me with the spores of Hypholoma and Coprinus, and to whose studies in the germination of the hymenomycetes I am indebted for the knowledge that they would be satisfactory for germination purposes in the media used.

have as nearly uniform a quantity of spores in the various cultures compared as possible. It is equally important to avoid bunches of spores in making the inoculation. Oversight in regard to either of these points is bound to result in more or less erratic and unreliable results. This is due to the fact that a toxic agent like copper, which effects the death of the spores by uniting with and precipitating the protoplasm, is itself also precipitated in the process and can no longer exert a deleterious influence. This precipitation process is naturally a strictly quantitative matter, and the amount of copper sufficient to sterilize a culture containing fifty spores may prove entirely inadequate were the number of spores quadrupled or even doubled. When bunches of spores are present the effect is still more mischievous. These bunches are relatively slowly penetrated by the copper-containing solution. Should the bunch contain air, as is usually the case, the process of penetration of the copper to the innermost spores is still further retarded. In the meantime such spores as are floating singly are quickly killed, precipitating their share of the copper, and it may be that long before the solution has fully penetrated the bunch it may have lost the greater part of its soluble copper and may be no longer able to kill the remaining spores. These may then germinate and grow almost as well as if no poison had been present. The difficulty was entirely overcome by soaking the spores for a longer or a shorter time in sterile distilled water with an occasional shaking. In some cases this soaking may be continued with advantage for days, in which case it is well to keep them at a low temperature to prevent germination. In all cases it was advisable to inoculate from a mixture of spores in water, a uniform inoculation being approximated in that way without great difficulty. It is equally important to have the culture drop of as uniform a size as possible. Absolute precision in these matters, however, is impossible, and the reader is reminded that slight variations in all cultures are to be expected. Indeed, as has been often suggested, spores taken from the same sporangium and placed in the same culture under absolutely similar conditions vary very

markedly in their capacity to resist poisons and other unfavorable conditions.

I would emphasize the importance of considering the *point of inhibition of germination* rather than the *death point* in consulting these results. The former can be determined with ease and absolute certainty by direct observation under the microscope, the latter only by transferring the spores to a fresh culture free as possible from the deleterious agent, and determining their success or failure to germinate under these conditions. The unavoidable source of error here is the transference of more or less of the copper compound with the spores. This quantity is doubtless often sufficient to prevent the germination of spores which although greatly reduced in vitality may be still alive. The general result of such transfers made at varying intervals from twenty-four hours to ten days indicated that if the germination of the spores be *inhibited* by a concentration of a copper salt, it was merely a question of time when they would be killed. Thus if a certain concentration inhibited the germination of a certain spore, possibly a solution of four times this concentration would fail to sterilize the culture in twenty-four hours, yet if left for some days the points of inhibition and death would finally coincide. The length of time necessary to bring this about would naturally vary with the varying specific characteristics of the spores of the different species.

The medium used for the cultures, the results of which are given in *fig. 1*, was a decoction of sugar beet. The root, sliced thin, in the proportion of 450^{gr} per liter, was heated in an autoclave for an hour at 120° C., under a pressure of one atmosphere. The decoction was then filtered and poured into flasks and thoroughly sterilized. This medium was selected for the comparative tests as giving the best results with the greatest number of the fungi used. The other vegetable decoctions were prepared in essentially the same manner.

Stock solutions of the various copper compounds were prepared by a responsible chemist. A *normal* solution of a copper compound means in this paper the number of grams of the salt

corresponding to the full molecular weight (*e.g.*, $CuSO_4 \cdot 5H_2O = 249$) dissolved in sufficient water to make one liter of solution; thus 0.01 *n* $CuSO_4$ equals a 0.249 per cent. concentration of the *crystallized* salt.

In the figures used to illustrate this paper the solid black bar indicates that the concentrations used were sufficiently strong to kill the spores within twenty-four hours. The square blocks alternating with the open spaces indicates that while the germination of the spores was inhibited, they still retained vitality enough to germinate several per cent. after twenty-four hours immersion. Four parallel lines indicate some germination but very poor development. Three parallel lines indicate fair but distinctly abnormal development, and two lines approximately normal germination and growth.

Fig. 1 shows in a condensed form the results of some four hundred cultures with fifteen fungi in a decoction of sugar beet to which had been added copper sulfate. A glance at the page will show that many of the fungi present a really remarkable similarity in resistance to copper. Twelve of the fifteen forms represent a range in lethal concentration of from 0.0099 *n* to 0.0168 *n*, or slightly less than 70 per cent. variation. This range of susceptibility may be modified by several important considerations, prominent among which is temperature. All, except Gloeosporium, were grown at 28° C., a little more or less. It is established that a fungus presents its greatest resistance to a deleterious agent, other things being equal, when cultivated at or near its optimum temperature for germination and mycelial development.[8] At a higher or lower temperature a smaller quantity of the agent results fatally. All the forms used, except *Gloeosporium malicorticus*, grew well at 28° C., but it is quite certain that some of them grow better at other temperatures. Inasmuch as the optimum temperatures for the different forms were unknown it was thought best to use a uniform

[8] CLARK, J. F.: On the toxic value of mercuric chlorid and its double salts. Jour. Phys. Chem. 5: 289–316. 1901; also FLENDE: Ueber Creolin und seine Wirksamen Bestandteile. Arch. f. Hyg. 9: ———. 1889.

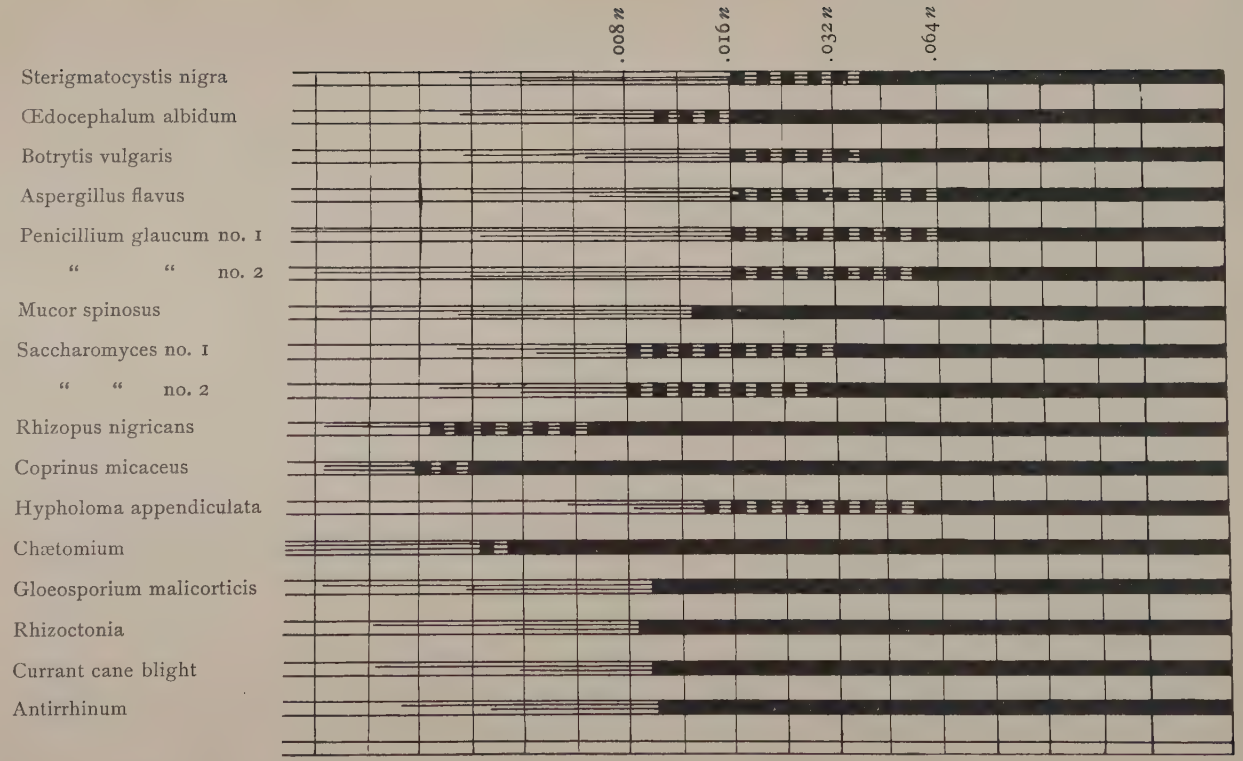

FIG. 1.

NOTE.—On account of the loss of the original drawings, while in the hands of the engravers, it is not possible to give *quantitative* values for this figure. The four values given are approximately correct. *Coprinus* was inhibited by .003 *n*.

temperature, except with Gloeosporium, which refuses to germinate at so high a temperature. It is also doubtless true that other *optimal* considerations have a similar effect in increasing the resistance of plants to unfavorable conditions. It should also be noted in studying these results that several of the forms were tested in media prepared at different times, and as sugar beets vary in composition the media would vary somewhat in nutritive properties, and therefore in toxicological properties also, when used as a solvent for a poison. Making due allowance for these considerations, however, it is perhaps remarkable that of a selection of fungi representing fairly well the natural groups and including typical saprophytic and parasitic forms, the great majority of them should be found to be killed under similar conditions by concentrations of copper of between 0.0099 *n* and 0.0168 *n*, and that the three forms which were found to be markedly more sensitive (Rhizopus, Coprinus, and Chaetomium) should be of such diverse relationships. The two species of yeasts proved to be equally resistant, although markedly different morphologically. The exact similarity of resistance was in this case very easily and prettily shown by growing both forms in the same culture drop. Such a test is applicable only where the forms are of similar properties as regards resistance to the agent, time of germination, and rapidity of growth. Two cultures of *Penicillium glaucum*, obtained from different sources and presenting slightly different habits of growth, also proved to be equally resistant.

The great variation between the two hymenomycetes is partly to be accounted for by the fact that the medium was very favorable for the development of Hypholoma, and only moderately suited for Coprinus. The similarity of resistance of the four parasitic forms (at bottom of figure) is quite striking. It will also be noted that they occupy an intermediate position. The most resistant forms will be seen to be the molds Aspergillus, Sterigmatocystis, Botrytis, and Penicillium.

Dr. Duggar has for some time been studying the broad field of food relation to toxicology in general. It has been

ascertained that fungi vary greatly in their resistance to deleterious agents, according to variations in the medium in which they are cultivated when submitted to the influence of the agent. His results have not yet been published, but the importance of this matter in any toxicological studies necessitates considerable reference to the subject in this study of the toxicology of copper.

The differences in resistance due to variations in the culture media may be due to two distinct causes: (1) All media influence the toxicology of electrolytes, and possibly other agents, by causing certain physical and chemical changes in the condition of the dissolved agent; (2) other things being equal, the more favorable the medium for germination and early mycelial development of a fungus, the more resistant that fungus will be to deleterious agents.

The simplest case of changed physical or chemical conditions would be the diluting of the solution of the copper salt with pure water. Here, with each decrease in concentration, we get less and less of the whole molecule, *e. g.*, $CuSO_4$, and more and more of its component ions, $^{+}Cu^{+}$ and $^{-}SO_4^{-}$, and the amount of change may be readily determined for any particular dilution. When the dilution is accomplished by adding an infusion or decoction of a vegetable or animal substance, however, a series of unknown reactions occurs, and even the amount of copper in the ionic form cannot be known. That these transformations have a very great influence on the toxic properties of the mixture is abundantly shown by the results represented graphically in *fig. 2*. A number of media tested caused precipitation of the copper, thereby rendering it harmless.[9] All such media were discarded and are not represented in the figure.

When spores are placed in a medium which does not admit of growth (*e. g.*, pure water) it is merely a question of time when these spores will be killed, if there be present a sufficient quantity of copper to precipitate the living proteids. It takes some

[9] COPELAND and KAHLENBERG: The influence of the presence of pure metals upon plants. Trans. Wis. Acad. 12: 454–474. 1900.

time, however, perhaps weeks, for all this copper to find its way into the spores. Should the medium be such as admitted growth, the production of protoplasm might easily offset its precipitation by the copper, and long before the copper could have its maximum effect the protoplasm would have increased manifold, and would require a corresponding increase in the concentration of copper to cause death. Thus it is that with every favoring influence the fungus can make a better fight for life.

The results given in *fig. 2*, with various media, and with copper in the form of the sulfate, are those with *Œdocephalum albidum*. The work repeated with *Rhizopus nigricans* gave essentially the same results. A single glance at the figure shows how much more toxic the copper proved to be when dissolved in pure water than when in any other medium, 0.0002 *n* being fatal in twenty-four hours. This is but one-fortieth of the concentration of copper required to give the same result in a decoction of sugar beet. When 1.5 per cent. asparagin was added to this beet infusion the toxic value of copper dissolved in pure water was to its value in the asparagin-beet medium as 142 : 1, and the comparison in the case of the medium compounded from asparagin and inorganic salts the ratio is 285 : 1. In this last case there was a slight precipitate noticed under the microscope, and it may be that some of the copper was precipitated by the phosphoric acid present. Exactly what proportion of this striking difference is to be assigned to the nutritive condition and what to chemical and physical transformations it is impossible to say. Inasmuch as the asparagin medium, while a good food for Œdocephalum, is not so good as beet decoction, we must concede that the remarkable decrease in toxic properties in the cases where asparagin is present is chiefly due to the physical and chemical changes. That these changes have a preponderating influence in the case of the beet decoction is also very probable. The experiment of diluting and concentrating the beet decoction seems to support this view. The values of the triple, the ordinary, the half, and the quarter strengths are not greatly different

.0014 n
.0018 n
.0024 n
.0031 n
.0040 n
.0051 n
.0067 n
.0087 n
.0011 n
.0015 n
.0019 n
.0025 n
.0034 n
.0045 n
.0058 n
.0075 n
.0098 n
.013 n
.017 n
.022 n
.028 n
.036 n
.047 n
.060 n
.079 n
CuSO4 in pure water
Decoction of Calla leaves
" " Allamanda leaves
" " pea seeds
" " celery stems
" " Pleurotus ulmarius
" " Lepiota naucena
Beef broth
Infusion of clover hay
Decoction of sugar beet
Beet dec. diluted to two vols. by pure water
" " four " "
" with 1.5% asparagin added
" concentrated to ⅓ volume
Decoction of bean seeds
Pure apple juice
Potato infusion
Prune decoction
MgSO4 5%; KH2PO4 1%; asparagin 2%; Fe2Cl6 trace

FIG. 2.

so far as stimulation of germination and early mycelial development is concerned. The same is true of the beet decoction to which the asparagin was added. Yet we have the following variation in the lethal concentrations:

Medium	Lethal concentration	Relative amount of Cu required to inhibit as compared with a pure water solution
Beet decoction, normal strength.....	0.0076 *n*	38 times
" " diluted to two volumes.....	0.0054 *n*	27 "
" " " " four "	0.0034 *n*	17 "
" " evaporated to ⅓ volume.....	0.0163 *n*	82 "
" " + 1.5 per cent. asparagin....	0.0284 *n*	142 "

Were it wholly a matter of food relation we should expect the figures in the third column to be near 38 in each case. Were it wholly a matter of the chemical or physical transformation of the copper by the compounds present in the beet decoctions, we should expect a more proportionate decrease in the first three numbers of the third column, corresponding to the dilution of the medium by the added pure water. As already remarked, it is not possible to determine with any degree of precision the part played by any one factor from the data at hand; but it is evident that with different media great differences in the toxic value of the copper may be obtained, and that asparagin has the power of uniting with salts of copper in such a way as to reduce their toxic properties in a very extraordinary manner. It is of interest in this connection to note that mercuric chlorid is not similarly reduced in toxic properties by the presence of asparagin.[10]

Fig. 3 presents the results of some studies on the addition of certain compounds to pure water solutions of some copper salts. Here the results given are with Œdocephalum, and, as in *fig. 2*, the results were checked over with *Rhizopus nigricans*. In this diagram the vertical lines representing the variation in the concentration of copper present in the different cultures show an increase of 30 per cent. for each division over the last preceding

[10] Jour. Phys. Chem. 5: 302. 1901.

Copper nitrate in pure water for 24 hours
Copper formate " " "
Copper acetate " " "
Copper chlorid " " "
" " " in 4% NH_4NO_3 sol. for 24 hours
" " " " Na_2SO_4 (?) " "
" " " " K_2SO_4 " "
" " " " KCl " "
Copper sulfate in pure water for 24 hours
" " " 5% grape sugar sol. for 24 hours
" " " 5% cane " "
" " " 4% glycerin " "
" " " 5% NH_4NO_3 " "
" " " 5% Na_2SO_4 " "
" " " 2% K_2SO_4 " "
" " " 5% " " "
" " " 5% KCl " "
" " " 3% K_2SO_4 for 10 days
" " " 2% pot.-sod.-tartrate for 10 days
.00023 n
.00030 n
.00039 n
.00051 n
.00066 n
.00086 n
.00112 n
.00146 n
.0019 n
.0024 n
.0032 n
.0041 n
.0053 n
.0069 n
.0087 n
.0114 n
.0148 n
.0192 n

FIG. 3.

division passing to the right. It will be observed that the five copper salts, the nitrate, formate, acetate, chlorid, and sulfate, dissolved in pure water have approximately the same toxic value, the variation (0.00004 *n*) being within the limits of experimental error. These five salts are fatal to Œdocephalum in 0.00020–0.00024 *n* concentration in twenty-four hours. This is about a 0.005 per cent. solution of the crystallized salt, $CuSO_4 \cdot 5H_2O$, or one part crystallized salt to 20,000 parts water.

The addition of glycerin, glucose, or cane sugar failed to cause any pronounced decrease in toxic properties. The addition of salts like K_2SO_4, KCl, etc., however, caused a very great reduction. One part $CuSO_4$ to 20,000 parts pure water is fatal to Œdocephalum in twenty-four hours. It requires sixty-one parts $CuSO_4$ to 20,000 parts of a 5 per cent. K_2SO_4 solution to have the same effect. That the lessening of the toxic value here was not due to a lessening of the rapidity of the penetration of the copper salt is shown by the last three lines of *fig. 3*, where the cultures after standing ten days showed a toxic value of less than one-fortieth that of the $CuSO_4$ in pure water. A test made by adding a small quantity of KCl to solutions of $CuCl_2$ gave an increase in the toxic value of the mixture. Further experimentation on this point is needed, but the indications are that here we have a similar phenomenon to that observed on the addition of chlorids to mercuric chlorid solutions,[11] small additions of a chlorid causing an increase, and larger amounts causing a decrease in toxic properties; the former probably through the formation of highly toxic $^{-}HgCl_4^{-}$ ions, and the latter through the formation of K_2HgCl_4 double salt molecules having a low toxic value. Copper reacts very similarly to mercury in respect to the formation of double salts with alkali chlorids.[12] These double salts would naturally be highly dissociated into metallic cations and the anion $^{-}CuCl_4^{-}$, and similar anions. That the ion $^{-}CuCl_4^{-}$ should have a high toxic value is very probable, being in harmony with all at present known regarding toxic

[11] CLARK, J. F.: Jour. Phys. Chem. 5: 289–316. 1900.

[12] REMSEN, IRA: Inorganic chemistry (advanced course) 589. 1892.

agents capable of uniting with more chlorin.[13] That the forcing back of the ionization of these double salts to the molecular form (*e. g.*, K_2CuCl_4) would result in a lowering of its toxic value seems more than probable, in view of what we know in the analogous case of the double salt K_2HgCl_4 already referred to, and furnishes a very reasonable hypothesis to account for the remarkable lowering of the toxic value of solutions of copper in pure water when a chlorid is added in excess. The decrease from the adding of sulfates, nitrates, etc. of the alkali bases is doubtless due to similar conditions.

Fig. 4 gives the results of a test of various soluble and insoluble copper compounds with three fungi grown in beet decoction. The letters *A*, *Œ*, and *B* refer to Aspergillus, Œdocephalum, and Botrytis respectively. Beet decoction has a very marked power of dissolving copper even in the metallic state, hence it was possible to test "insoluble" copper compounds, such as Paris green, copper hydroxid (the form in which the copper is found in Bordeaux mixture), etc. This power of dissolving copper is a property of almost all decoctions of vegetable or animal substances, some possessing it in a much higher degree than others.

Fig. 4 shows that copper, gram for gram, has much the same value toxicologically when added to a beet decoction, no matter what form of copper salt is used. The apparent exceptions to this are are to be accounted for by the effect of the other atoms and groups added. Thus the chromate salt which proved to be by far the most toxic compound of copper tested owes its high toxic value to the deadly chromium contained.[14] The *eau céleste* and the ammoniacal solution of copper carbonate are particularly fatal to Œdocephalum and Botrytis on account of the basic alkalis present.[15] Œdocephalum is apparently very sensitive to compounds containing arsenic. Perhaps it is especially sensitive to this element, but so far as I know the point has not been tested. That the green arsenoid should be more fatal

[13] DAVENPORT, C. B.: Experimental morphology 8. 1897.

[14] CLARK, J. F.: Bot. Gaz. **28**: 390, 396. 1899. [15] *Ibid.* **28**: 380–382. 1899.

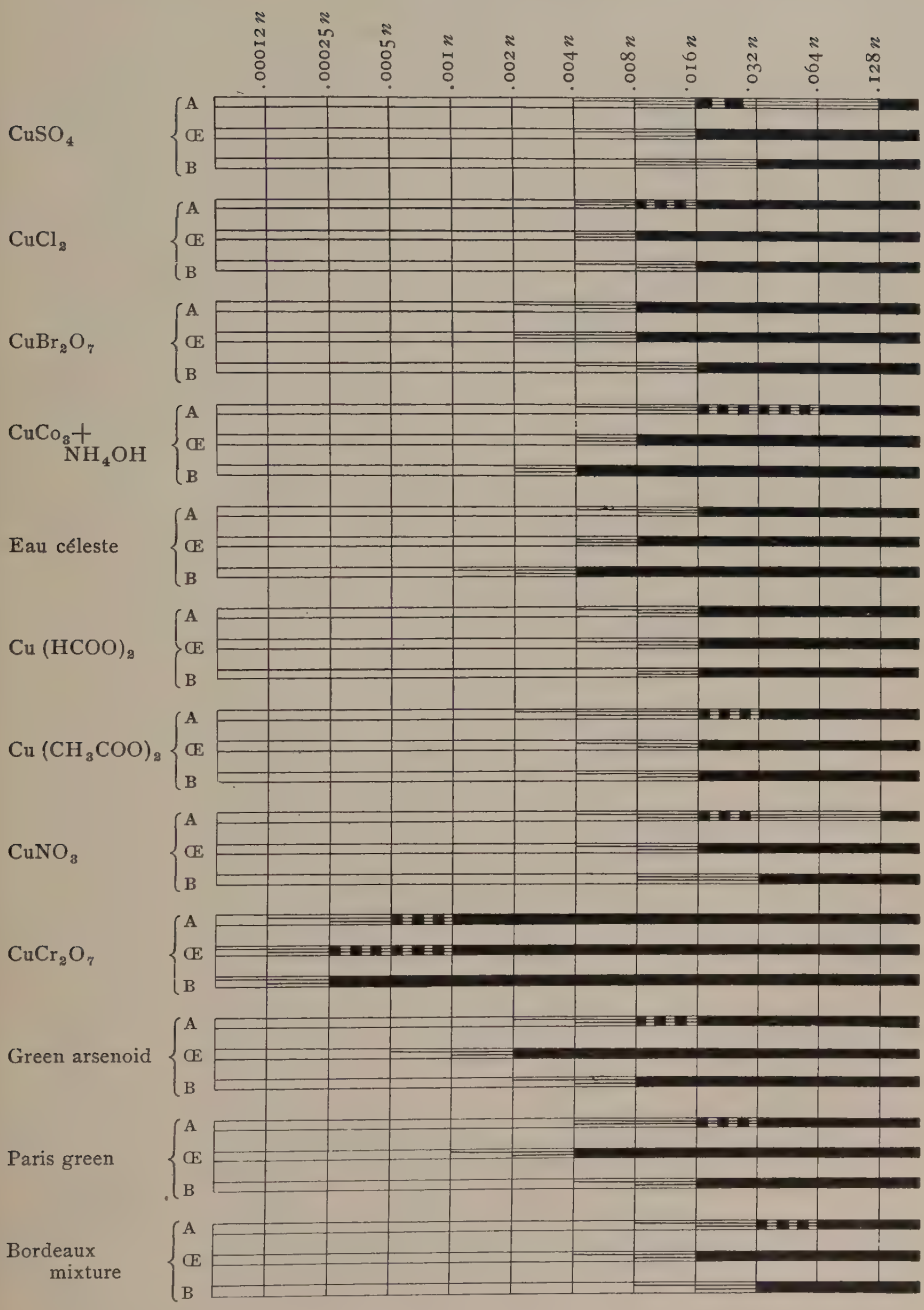
.00012 n
.00025 n
.0005 n
.001 n
.002 n
.004 n
.008 n
.016 n
.032 n
.064 n
.128 n
CuSO4
CuCl2
CuBr2O7
CuCo3 + NH4OH
Eau céleste
Cu (HCOO)2
Cu (CH3COO)2
CuNO3
CuCr2O7
Green arsenoid
Paris green
Bordeaux mixture
A
Œ
B

FIG. 4.

than the Paris green was to be expected, inasmuch as it showed on analysis 8.3 per cent. soluble As_2O_3 as compared with 1.8 per cent. soluble in the sample of Paris green. That the ammoniacal solution of $CuCO_3$ is a more effective fungicide than Bordeaux mixture for the amount of copper used does not follow from the showing made in this figure. These tests were made in closed cells where the excess of ammonia was indefinitely retained, whereas under orchard conditions this evaporates in a few hours at most, and exerts practically no fungicidal value.

It would be beyond the province of the present paper to give a detailed account of my experiments on the toxicology of the Bordeaux mixture. These are of greater interest to horticulturists than to botanists. I shall mention briefly, however, the more important experiments and state the conclusions arrived at from this study.

Just how the insoluble copper hydroxid of the Bordeaux mixture sprayed on leaves has protected them so thoroughly from the ravages of parasites has long been a mystery to the chemist and physiologist alike. Various hypotheses have been propounded from time to time, but up to the present no satisfactory explanation has been given. A very careful review of the current hypothesis may be found in Swingle's paper.[16] His own suggestion that it might be possible that the fungi themselves, by secretions or excretions, might be able to aid in the solution of the copper has proved a happy one. As already mentioned, most animal and vegetable decoctions have a greater or less power of dissolving insoluble copper compounds.

This power is particularly noticeable in the fungi. An infusion of *Agaricus campestris*, and infusions and decoctions of various parasitic fungi, dissolve copper hydroxid very quickly and in sufficient quantity to inhibit the germination of any fungus tested. Spores of various fungi placed in a mixture of $Cu(OH)_2$ and water were killed in a short time, while other spores of the same species, placed in similar mixtures of $Fe(OH)_2$

[16] SWINGLE, W. T.: Bordeaux mixture. Bull. no. 9. Div. Veg. Phys. and Path. U. S. Dept. Agric. 1896.

and $Al(OH)_3$ retained their vitality unimpaired for weeks. The death of these spores was not due to the presence of any soluble copper, for no copper could be detected in the filtrate by the most delicate chemical tests before the spores were added. Traces of copper were found some time after the addition of the spores (if added in considerable quantity) showing that they dissolve more of the $Cu(OH)_2$ than was required to kill them. The rapidity with which fungus spores are thus killed naturally varies with the character of the contents and coverings of the spores. It is very quickly accomplished in thin-walled spores, such as Rhizopus and Œdocephalum, but is very slowly accomplished in forms like Aspergillus and Penicillium, whose coats present great resistance to the entrance of poisons and the excretion of copper-dissolving compounds. As an example of such resistance to entrance, see experiments with Aspergillus in solutions of nickel sulfate,[17] in which $0.03\,n$ inhibited germination, but immersion in a normal solution (containing over 13 per cent. $NiSO_4$) for 48 hours at 28° C. failed to kill the spores. The same form resists immersion in double normal $ZnSO_4$ (27 per cent. anhydrous $ZnSO_4$) under similar conditions.

The host plant, too, is active in dissolving the $Cu(OH)_2$. A peach tree was sprayed with Bordeaux mixture which contained an excess of lime, as is ordinarily the case. Following this, the tree was sprayed several times during the day with enough pure water to have an effect resembling dew. The following day water collected from the leaves, filtered and evaporated to small volume, showed just a slight copper reaction. Several small leafy branches were then removed and placed in a large beaker of distilled water, without injury to any of the parts. After soaking for some hours these were removed and the solution filtered, evaporated to small volume, and tested for copper. A marked reaction was secured, indicating the presence of considerable copper in solution. The epidermis of leaves, although protected by a cuticle, is well known to be more or less permeable to the dissolved substances occurring in the cell sap, particularly along

[17] CLARK J. F.: Bot. Gaz. 28: 392. 1899.

the union of the epidermal cells.[18] When the dew is on the leaf we have two solutions—the dew drop without and the cell sap within—separated by a more or less permeable membrane. The result of these conditions must result in the exosmosis of at least some of the contents of the cell sap, which coming in contact with the copper hydroxid adhering to the leaf surface causes more or less of it to pass into solution. An important point in this connection is that the solution of the copper, whether effected by the host or the fungus spore itself, is possible in the presence of an excess of lime. This excess is apparently somewhat detrimental to the solvent action on the copper, and in this way doubtless serves a very valuable purpose in preventing a too rapid solution. It also protects the $Cu(OH)_2$ from the solvent action of the ammonia, nitrites, and nitrates of the atmosphere. According to Millardet and Gayon's[19] experiments, the amount of lime ordinarily used in Bordeaux mixture is sufficient to protect the copper absolutely from solution for eighteen days under ordinary atmospheric conditions. This ought to have been sufficient to have set aside the hypothesis which accounted for the solution of the copper of the Bordeaux mixture by atmospheric conditions, for all experiments go to show that the Bordeaux mixture is effective from the day it is applied. So much in brief review of some of the experiments that have led me to the following conclusions regarding the toxicology of this invaluable fungicide:

1. The solution of that part of the $Cu(OH)_2$ of Bordeaux mixture which under orchard conditions *is of fungicidal value, is chiefly accomplished by the solvent action of the fungus spores themselves*, for they have power to dissolve sufficient copper to kill themselves.

2. The amount of copper necessary for the destruction of the spores of parasitic fungi is probably not more than one part of soluble metallic copper to 80,000 parts water (dew).

[18] Büsgen: Ueber einige Eigenschaften der Keimlinge parasitischer Pilze. Bot. Zeit. 57: 67. 1893.

[19] Millardet et Gayon: Les divers procédé de traitements du mildiou par les composés cuivreux. Jour. des Agr. Prat. 1: 701–702. 1887.

3. The host plant has a greater or less power of dissolving the $Cu(OH)_2$ deposited on its leaves.

This solution of the copper by the host plant may or may not be advantageous in protecting it from attack, as injurious spores may or may not chance to be present at the point where solution has taken place. To this factor, however, must be attributed the injury frequently observed with certain plants (*e. g.*, Japanese plums, peach, etc.) when sprayed with Bordeaux. The amount of injury done to a given species will depend on the following considerations, provided always that carefully prepared Bordeaux mixture has been used:

1. The *specific susceptibility* of the protoplasm of the plant to poisoning by copper.

2. The *solvent properties* of the cell sap on copper hydroxid.

3. The *permeability of the epidermis or cuticle*[20] to these cell contents when the conditions are favorable for their exosmosis, and for the entrance of the copper after its solution is effected.

4. Weather conditions following spraying, particularly conditions as regards moisture (dew, etc.), providing the conditions for the exosmosis of some of the contents of the cells of the leaf.

To these might be added, perhaps, climatic conditions which, through modifications of structure, of specific resistance, or of the character of the cell sap, may cause a plant to be more or less resistant to treatment with Bordeaux mixture, according to the locality where it is grown. The very injurious influence of Bordeaux mixture on blossoms[21] is to be explained in an entirely similiar way. Here the protection of the cells by cutinization is almost or entirely wanting, and it is very probable that the rich

[20] Dr. W. C. Sturgis (Rep. Conn. Agric. Exper. Sta. 219–254. 1900) has done some very careful work on the effect of Bordeaux mixture on peach foliage. In his study for a cause of the extreme susceptibility which he found in his peach trees, he has unfortunately overlooked the possibility of specific sensitiveness to copper poisoning of the protoplasm; and in the study of the structure of leaves the study of the cutinization of the epidermis has been omitted. It is hardly possible that the aeration of the spongy parenchyma can be a factor in the case.

[21] BEACH, S. A., and BAILEY, L. H.: Fruit-setting of sprayed blossoms. Bull. 196. N. Y. Agric. Exper. Sta. 1900.

contents of pollen grains and the cells of the stigma have an unusually solvent action on the copper hydroxid.

Swingle's proposition that the copper might be effective in preventing the attack of parasites through negative chemotactic action induced me to make a number of experiments on this point. A number of fungi were used to a greater or less extent,

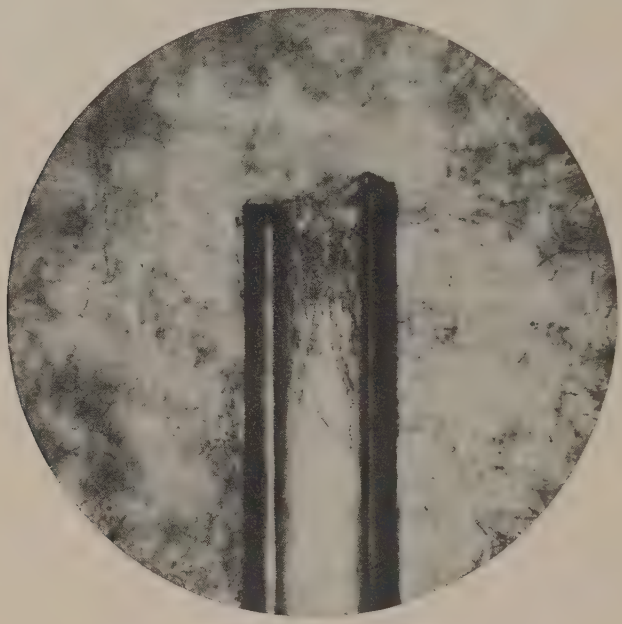

FIG. 5.

but Rhizopus proved the most satisfactory, inasmuch as it usually germinates with a single germ tube and grows rapidly with a minimum of branching.

The well-known method of Pfeffer was first employed. Capillary tubes closed at one end were filled with solutions of copper of varying strengths. Hanging drop cultures of the fungi were prepared in weak agars of varying consistence. Into these hanging drop cultures were introduced the open ends of the copper-containing capillary tubes. These tubes were introduced at different stages of germination. The uniform result of

these preparations was that the hyphae near the mouth of the tube grew into the solution of copper, and continued to grow directly inwards until they reached a concentration sufficient to kill them (*fig. 5*). This was found to be the case with copper, cobalt, nickel, zinc, etc., and *also when the capillary tube contained pure water.*

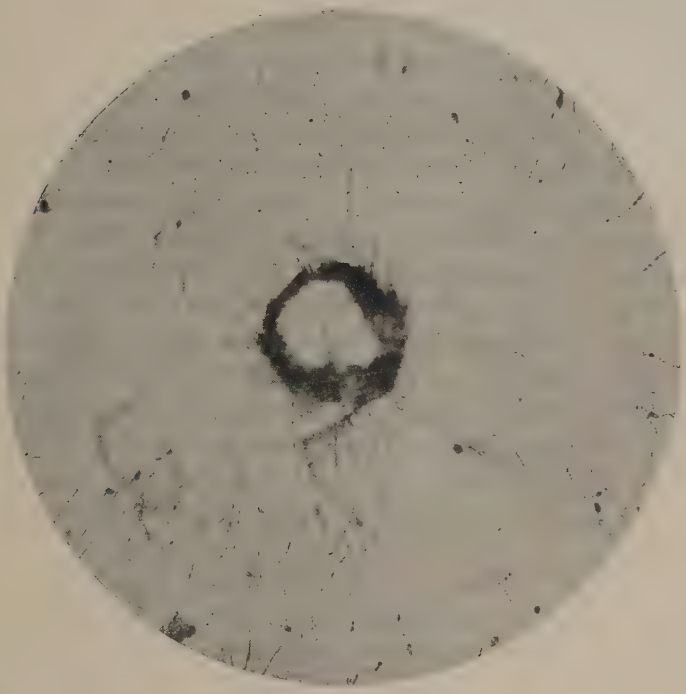

FIG. 6.

Miyoshi's[22] method of injecting leaves with solutions by placing them in the solutions under an air pump and exhausting was next tried. After the injection of the leaves they were washed under the faucet and spores of Rhizopus were sown on the lower surface. The preparation was then placed in a moist chamber in an incubator at 28° C. The germ tubes near stomata curved towards and grew directly into them in a manner quite similar to what Miyoshi found to be the case when the leaf was

[22] MIYOSHI, M.: Ueber Chemotropismus der Pilze. Bot. Zeit. **52**: 1–27. 1894.

injected with food solutions. In many cases upwards of thirty germ tubes were counted growing into a single stoma. On injecting similar leaves with pure water, however, I got a similar result. The leaves used were chiefly from *Tradescantia discolor*. The concentrations of copper, cobalt, etc., injected ranged from 0.01 to 0.005 *n*.

I then tried Miyoshi's method of using perforated mica plates with layers of media between. Spores were sown in every conceivable way: in upper, middle, and lower layers; in nutrient and non-nutrient media; and in media containing copper. The uniform result of these tests was that the germ tubes near the perforations grew towards the opening *if it communicated with a layer of media in which no spores had been placed*. The presence or absence of copper was not found to have any influence on the *direction* of growth. The hyphae grew towards, and in cases where the concentration of copper was not too great, grew through the perforation into the copper-containing medium. *Fig. 6* illustrates such a case. The spores of Rhizopus were sown in a rich nutrient agar. Upon germination they immediately turned towards a perforation in the mica plate which communicated with a non-nutrient agar impregnated with 0.005 *n* $CuSO_4$.

Finally, I prepared a series of cultures in large Van Tieghem cells 5^{cm} in diameter. The nutrient agar in which the spores were placed occupied the part marked *a* in *fig. 7*. The portions of agar marked *b b* were prepared from nutrient or non-nutrient agar to which varying concentrations of copper had been added. The portions *c c* are checks, being duplicates of *b b* without the copper. The inoculation was made by placing a few spores of Rhizopus at the center of *a*. The cultures were then placed in the incubator at 28° C. until the fungus had made considerable growth. The cells were then opened and *b b* and *c c* were pushed towards *a* until in slight contact and occupying the positions *b' b'* and *c' c'*. More or less of the Cu in solution in *b'* and *b'* would then pass into *a*, and if the fungus was either attracted or repelled by the Cu it would be readily determined by watching

the behavior of the hyphae as they grew down *a* towards the extremities *d d* between the copper-containing portion *b'* and the check preparation *c'*. The hyphae did curve towards *b'* and *b'*, but also towards *c'* and *c'*, and the *larger* the portions *b'* or *c'*, the more strongly they curved towards them. This curving took place even when *b* and *c* contained no nutrient substance, the hyphae growing from the nutrient portion *a* into a nonnutrient medium. Cultures on this plan were repeated many times and varied in many ways, *e. g.*, varying the consistence of the agar, the nutrients used, the concentration of copper, etc.

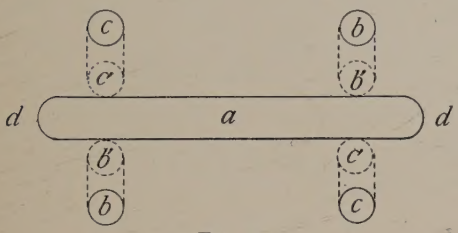

FIG. 7.

My interpretation of these results is that Rhizopus is markedly negatively chemotactic to some secretion of its own mycelium, and this negative chemotropism is much greater than any positive chemotropism it may have for food substances or oxygen. This method then, which seems to be satisfactory, has failed to show that Rhizopus has positive or negative chemotropism towards copper or allied metals. These cultures were used to a less extent with several other saprophytic and two parasitic forms, with the same result in regard to chemotropism towards copper. A large number of photomicrographs and drawings with camera lucida were made, but those reproduced in *figs. 5* and *6* are as instructive as any.

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